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Acute Respiratory and Systemic Responses to Diesel Exhaust Particle Exposure

By

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Abstract

For decades, epidemiological studies have shown increases in morbidity and mortality associated with exposure to particulate air pollution. Increasingly, experimental data is unravelling the precise mechanisms through which particulate matter exerts its effects on the aggravation of respiratory and cardiovascular conditions. We undertook a series of *in vivo* studies to measure the acute effects of diesel exhaust particles (DEP) on pulmonary and systemic inflammation, by administering DEP to Balb/c mice intranasally. We observed significant neutrophil and antigen presenting cell infiltration into the lungs after exposure to DEP. Significant increases in CD54 expression suggest that DEP had a maturation effect on dendritic cells (DC). Increased production of GM-CSF in association with DEP treatment was also shown. Systemic inflammation was demonstrated by a significant increase in serum amyloid A (SAA) in association with DEP treatment; interestingly, no concomitant increases in fibrinogen or sP-selectin were observed. These results indicate that significant pulmonary inflammation and partial induction of the systemic response is linked to exposure to DEP. Pulmonary inflammation and DC maturation are implicated in aggravation of allergic respiratory symptoms and morbidity; increased SAA in serum is a known marker of cardiovascular risk. While further study is needed, these results contribute to our current understanding of the impact of inhaled pollutants on respiratory and cardiovascular health.

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Abbreviations

Ag	antigen
AP-1	activator protein 1
APC	antigen presenting cell
BAL	bronchoalveolar lavage
CD	cluster of differentiation
CRP	c-reactive protein
DC	dendritic cell(s)
DEP	diesel exhaust particles
ELISA	enzyme-linked immunosorbent assay
HBSS	Hank's Balanced Salt Solution
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour
mg	milligrams
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
MNC	mononuclear cell
NF-κβ	nuclear factor kappa beta

ng	nanogram
PCBs	polychlorinated biphenyls
PM	particulate matter
ROS	reactive oxygen species
SAA	serum amyloid A
TCN	total cell number
U	unit
VOCs	volatile organic carbons
µg	microgram
µl	microlitre
µm	micrometre

Introduction

For decades, epidemiological studies have demonstrated a link between air pollution and morbidity and mortality. The World Health Organization suggests that the burden of disease associated with air pollution is most strongly felt in developing nations. In fact, the highest levels of air pollution are found in low and middle-income countries. At the same time, evidence indicates that there is no known safe threshold for air pollution exposure (World Health Organization, 2006). In 2008, the Canadian Medical Association estimated that 21,000 premature deaths and over 20 million minor illnesses were linked to poor air quality in Canada (Canadian Medical Association, 2008). According to the study, those at highest risk of morbidity and mortality due to air pollution are children and the elderly. Children are at increased risk because they inhale a larger volume of air for their body weight compared to adults, resulting in a greater concentration of inhaled pollutants. Elderly are often at increased risk due to existing medical conditions, such as respiratory and cardiovascular disease. As the population in Canada ages, and if air pollution is not reduced, morbidity and mortality linked to air pollution is expected to rise dramatically (Canadian Medical Association, 2008).

Air pollution is the contamination of the air by particles and gases in amounts that are harmful to living things. A proportion of air pollution originates from natural sources, such as forest fires, windblown dust, and pollen release. Human activity also

makes a significant contribution to air pollution, including emissions originating from motor vehicles, the combustion of fossil fuels, and industrial processes (pulp and paper mills, ore refineries and petroleum processing). Emissions released are often a heterogeneous mix of particles and gases (Health Canada, 2006). For instance, automotive exhaust is composed primarily of gases such as nitrogen oxides, carbon monoxides, and particulate hydrocarbon compounds, metals and soot (United States Environmental Protection Agency, 2009). Some of the most commonly measured gaseous air pollutants include ozone, carbon monoxide, sulphur dioxide, and nitrogen oxide. Some of the most commonly measured particulate pollutants include toxic metals (lead, nickel, mercury, cadmium, and manganese), volatile organic carbons (VOCs), polychlorinated biphenyl's (PCBs), benzene and formaldehyde (Health Canada, 2006).

The particulate portion of air pollution is often categorized by particulate size. Particulate matter (PM) $\leq 40\mu m$ in diameter will remain airborne, and thus contributes to the contamination of the air. PM ≤ 10 can be inhaled into the human respiratory system. Fine particulates ≤ 2.5 μm in diameter are more dangerous than larger particles because they have the capacity to remain airborne for prolonged periods of time, and are easily inhaled deep into the lungs where they can exert toxic effects (Health Canada, 2006).

The chemical composition of particulate matter varies by factors such as origin, weather, regional and local influences (Donaldson, 2007). Traffic density, industrial activities, domestic heating (by wood burning or fossil fuels) and population density are implicated in differences in ambient particulate matter from site to site (Donaldson, 2007). In urban areas, ambient particles are largely composed of organics derived from the combustion of fossil fuels such as gasoline and diesel (Diaz-Sanchez, D., 1997).

Particles generated in industrial areas, such as those from smelting and mining processes, are often rich in metals and acidic sulphates (Donaldson, 2007). Fine particulates are known to be predominantly generated by complex reactions between particles and gases in the atmosphere (Ministry of the Environment, 2009). Concentration and composition of PM in a given location is also shown to vary due to transportation of particles over long distances in the atmosphere (Ministry of the Environment, 2009). As a result, it is possible to be exposed to toxic particles generated by industries hundreds of kilometres away.

1.0 Respiratory Diseases and PM

In 1952 in London, UK, a heavy fog laden with pollutants from stoves and industrial processes held over the city of London for several days. During this time, there was a 48% increase in all hospital admissions and a staggering 163% increase in admissions related to respiratory disease. In the three months following the event, approximately 12,000 deaths were associated with exposure to the polluted fog (Simkhovich, B.Z., 2008). This event, and others like it in other European countries and in North America, brought the dangers of inhaled pollutants into focus and motivated research that has since demonstrated the clear links between particulate matter and respiratory illness.

Recent research has clearly described effects of air pollution on respiratory health. Health researchers in the city of Toronto, Canada, estimate that 1700 premature deaths and 6000 hospital admissions are related to air quality each year (Toronto Public Health, 2004). In Vancouver, Canada, hospital admissions for respiratory diseases increased

4.4% in elderly individuals on days of poor air quality (Fung, K., 2006). A study looking at communities located near to the Canada-U.S. traffic crossing in Buffalo, New York, found that asthma symptoms were reported 4 times more often in residents living near the traffic crossing versus kilometres away (Oyana, T.J., 2004).

Particulate matter pollution is a significant contributor to respiratory morbidity. A study out of Ontario, Canada, showed that over a 6-year period of observation, an average increase of 13 $\mu g/m^3$ in ambient particulate matter was significantly associated with 3.7% increase in hospital admissions for respiratory diseases (Burnett, R.T., 1995). A short term study of elderly individuals found that a 25 $\mu g/m^3$ increase in ambient particulate matter was associated with a 10.9% increase in respiratory mortality (Jiménez, E., 2009). Particulate matter is implicated in aggravations in asthma and subsequent hospital visits. For instance, a 1.1 $\mu g/m^3$ increase in traffic-related particulate matter was associated with 7.8% increase in hospital admissions for asthmatics (Halonen, J.I., 2008). Additionally, aggravations in allergic rhinitis and asthma, including symptoms such wheezing, mucous production and increased use of medication are shown to be linked to particulate matter exposure (Damato, G., 2010). These and other studies clearly indicate that particulate matter is a risk factor for respiratory health (Mar, T. F., 2010; Mamessier, E., 2006; Salvi, S., 1999; Slaughter, J. C., 2005; Yeatts, K., 2007; Sydbom, A., 2001).

2.0 Cardiovascular Diseases and PM

In addition to respiratory and immune responses, exposure to air pollutants, including particulate matter, is associated with cardiovascular morbidity and mortality. Particularly in high-risk groups such as those with pre-existing inflammatory conditions

and the elderly, indexes of cardiovascular health are significantly impacted by air pollution exposure (Simkhovich, B.Z., 2008). Even in middle-aged cohorts under the age of 65, troubling statistics show that risk of myocardial infarction is increased almost 10% in people living in urban, high-traffic areas versus areas with considerably less pollution (Tonne, C., 2007). The effects of particulate matter on cardiovascular health are also becoming well established. Significantly, Burnett et al (1995) showed that an increase in ambient particulate matter of 13 $\mu g/m^3$ was linked to an increase of 2.8% in hospital admissions for cardiovascular conditions in Ontario (Burnett, R.T., 1995). A study out of Los Angeles, CA, found that a 10 $\mu g/m^3$ increase in ambient particulate matter was associated with 6% increase in carotid artery intima-media thickness (a measure of atherosclerosis). Of individuals taking cardiovascular medication, the increase was found to be nearly 16% (Kunzli, N., 2005). An interesting study from Tokyo, Japan, associated particulate matter pollution with deaths by myocardial infarction: within 6 h of particulate matter pollution reaching peaks between 100-300 $\mu g/m^3$, the incidence rate of death by heart attack increased by 20% when compared to levels during episodes of improved ambient particulate concentrations (0-99 $\mu g/m^3$) (Murakami, 2005). Particulate matter is clearly associated with risk to cardiovascular health, as shown in these and other studies (Baccarelli, A., 2008; Brook, R.D., 2004; Donaldson, K., 2001; Lucking, A.J., 2008; Peters, A., 2001).

3.0 Toxicity of Particulate Matter Pollution

The toxic physiological effects of inhaled particulate matter are postulated to be due to the generation of oxidative stress by reactive oxygen species (ROS) (Donaldson,

K., 2003). ROS are highly reactive molecules including oxygen ions, free radicals and peroxides which can exert effects on cells such as protein oxidation, lipid peroxidation and DNA damage. While it is not completely understood how ROS are produced after inhalation of particulate matter, it is known that components on the particle surface react with molecules on the cell surface generating ROS. For instance, Aust et al (2002) showed that metals on the surface of particulates were capable of catalyzing redox reactions in the lung epithelia, generating ROS (Aust, A., 2002). Other reactions observed to generate ROS include the metabolism of polyaromatic hydrocarbons and quinones on the particle surface (Wan, J., 2007).

Significantly, studies show that particulates with different chemistry and from different sources have varied toxic effects. Aust et al (2002) showed *in vitro* that metal content was a significant contributor to inflammation after exposure to various particles resulting from combustion of coal fly ash, and diesel and gasoline engines. Additionally, in a study by Medeiros et al (2004), in comparison to pure carbon particles, particles sourced from a steel plant in Brazil had significantly increased toxic effects in the lungs. An interesting study from the Netherlands linked respiratory allergy inflammatory effects to particles sourced predominantly from traffic, industrial combustion and incinerators and not crustal materials or sea spray. Therefore, depending on the source and composition of particles, the toxic effects are known to be varied (Aust, A., 2002; Steerenberg, P.A,. 2006; Schwarze, P.E., 2007; Singh, P., 2004; Medeiros, N., Jr., 2003).

Normally, the potentially damaging effects of reactive oxygen species in the body are neutralized by antioxidants. In conditions where ROS are abundant, however, redox homeostasis can become overwhelmed resulting in oxidative stress. Under these

conditions, redox sensitive transcription factors activator protein 1 (AP-1) and nuclear factor kappa beta (NF-κβ) are induced (Donaldson, K., 2003). These factors initiate the transcription of genes that bring about the production of pro-inflammatory cytokines, chemokines and adhesion molecules (Li, N., 2003).

3.1 Local Inflammation after PM Exposure

The cells of the airway epithelia and are some of the first to respond to PM after inhalation. These cells are implicated in the secretion of pro-inflammatory mediators induced by conditions of oxidative stress. Cytokines such as GMCSF, TNF-α, interleukin 8 and interleukin 6 are secreted by bronchoepithelial cells in response to PM (Salvi, S., 1999; Takizawa, H., 2004). These cytokines act in the recruitment and activation of inflammatory cells like neutrophils and macrophages. Neutrophils are phagocytic cells that are among the early responders to the site of inflammation. They contain enzyme granules that are used to break down foreign material or damaged tissue. Macrophages are phagocytic cells that are residents in pulmonary tissue. Their numbers are increased in the tissue in times of inflammation. Along with epithelial cells, macrophages secrete pro-inflammatory cytokines that prompt the recruitment and activation of immune cells such as antigen presenting cells (APC) and lymphocytes (Abbas, A. K., 2007). The activation of immune cells has implications for respiratory diseases such as allergy and allergic asthma.

3.2 Effect of PM on APC and Adaptive Immune Responses

The activation of immune cells after PM exposure propels the adaptive immune response to respond vigorously to antigens. APC resident in the airways play a significant role in the initiation of immune responses. Antigen presenting cells, specifically dendritic cells (DC) and macrophages, continuously sample, process, and present inhaled antigens. Macrophages and dendritic cells have the ability to present antigens to T-cells to initiate the adaptive immune response (Abbas, A. K. 2007), but DCs are the most potent antigen presenting cells for naïve T cells because they have a more potent and precise strategy for antigen presentation than any other APC. The presentation of MHC-antigen peptide complexes is 10-100 times higher on the DC surface than on any other APC (Banchereau, J., 1998). Also, dendritic cells migrate to the lymph nodes in order to present antigens to populations of T-cells with greater efficiency (Abbas, A. K., 2007).

Dendritic cells arise from bone marrow progenitors. After transport through the blood, these DC precursors home to lymphoid tissues such as the lymph nodes and spleen, and also to strategic areas of the peripheral tissues, such as the epithelia and the airways (Sabatte, J., 2007). Of particular interest to us are the DCs in the airway epithelia that may interact with inhaled particulate matter.

DCs have a stellate shape characterized by processes extending off the cell body. These processes can fit between nearby cells, and enhance the capacity of the DC to survey the nearby environment for signs of threat (Banchereau, J., 1998). The DC will remain in a resting state until it is stimulated by an encounter with an inhaled Ag or

inflammatory cytokines. At this point, the dendritic cell will mature and migrate to the lymph node.

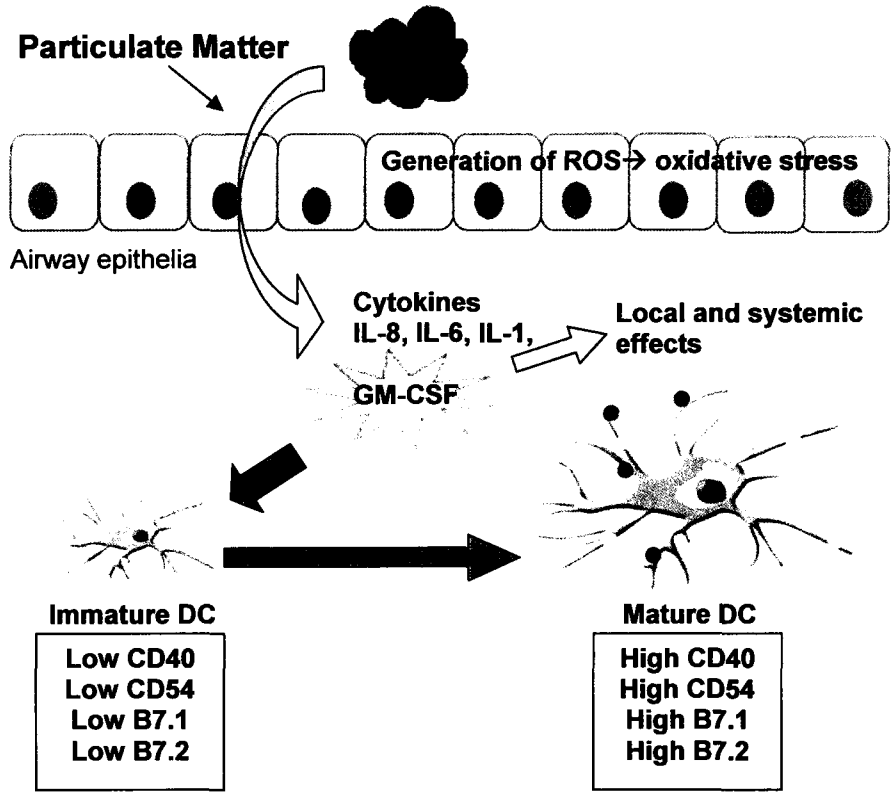

Figure 1:

Effect of particulate matter on airway epithelia and maturation status of DC.

3.2.1 Dendritic Cell Maturation and T cell Stimulation

A mature dendritic is characterized by the expression of a set of cell surface markers that assist in the initiation of an immune response. These molecular markers include CD40, CD54, B7.1 and B7.2. B7.1 and B7.2 are structurally and functionally similar glycoproteins often referred to as 'B7 molecules' (Abbas, A. K., 2007). The

ability of a dendritic cell to initiate the adaptive immune response by T cell activation is dependent upon two signals, one from a bound Ag (MHC-peptide complex) and a second co-stimulatory signal from B7.1 or B7.2. Without the interaction of both an Ag and one of the B7 molecules, the T cell will not become activated toward the presented Ag. The cell surface marker CD40 is an early activation marker on the DC, and has the ability to interact with the CD40 ligand of the T cell. When CD40 is stimulated by the T cell, signals are transmitted to the DC to enhance expression of B7 molecules. CD54 is an adhesion molecule that assists in prolonging the interaction between the DC and the T cell. This adhesion provides time for the T cell to survey antigens presented by the DC for the Ag specific to the T cell (Banchereau, J., 2000).

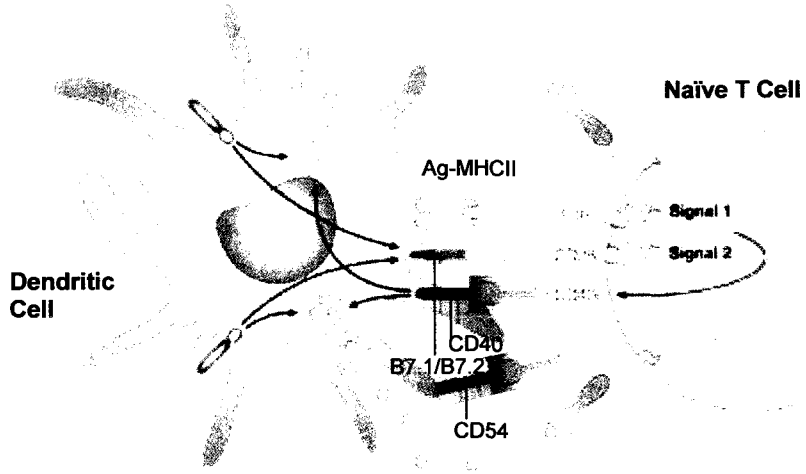

Figure 2:

Dendritic cell and T cell interacting.

3.2.2 Dendritic Cells, T cells and Allergy

A dendritic cell that captures an antigen will mature and migrate to the lymph nodes to present the antigen to T cells. T cells in the lymph node interact with DC to investigate the antigens presented. Each T cell is designed to initiate a specific immune response toward one specific antigen. When the T cell and the presented antigen make contact, and always in combination with appropriate co-stimulatory molecules, the T cell becomes activated toward the antigen. From here, the T cell will expand into a population of clones capable of mounting an immune response against the antigen (Janeway, C.A., Jr., 2005). In the case of the allergic immune response, the cloned T cells will be capable of activating B cells to produce IgE antibody against the antigen. The IgE antibody binds to cells such as mast cells and basophils, and gives them the ability to react to the antigen. When mast cells and basophils recognize the antigen, the response includes the release of histamine, enzymes, leukotrienes, platelet activating factor, prostaglandins and cytokines. The physiological effects of these secretions are considered hallmarks of allergy, such as bronchoconstriction, secretion of mucus, smooth muscle contraction, vasodilation, and increased vascular permeability (Abbas, A.K., 2007). Given the key role of DCs in initiating this process, any stimulus that can potentiate DC activity may be able to trigger allergic sensitization; thus, the effect of inhaled particulate matter on airway-resident DCs in the development of respiratory allergic diseases, particularly asthma, is a key focus of the current work.

3.3 Induction of Acute Phase Response

After exposure to particulate matter, it is possible for local inflammation to progress to a systemic response (Mukae, H., 2001; Terashima, T., 1997). The acute phase response is a systemic response in part initiated by the effects of inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Abbas, A.K., 2007). It is characterized by the shift of production of inflammatory mediators by the tissues to the synthesis of proteins by the liver. The proteins synthesized by the liver during the acute phase response are called acute phase reactants and include C reactive protein (CRP) and fibrinogen (Abbas, A.K., 2007). The prothrombotic effects of systemic inflammatory mediators are theorized to have adverse impacts on cardiovascular health, such as increases in the generation and rupture of atherosclerotic plaques, increased thrombus formation, increased plasma viscosity and increased blood pressure (Mills, N.L., 2009).

3.3.1 CRP and Serum Amyloid A

CRP is produced by the liver and released into the blood under conditions of inflammation, tissue injury and infection. CRP functions to bind to bacteria, induce activation of the complement system, and enhance phagocytosis. CRP has no known direct impact on blood coagulability. However, CRP is a well established independent marker of cardiovascular risk due to being a clear indicator of inflammation (Peters, A., 2001). Epidemiological studies show that increased levels of CRP are strongly associated with particulate matter pollution (Pope, C. A. III, 2004). The link between PM and CRP does not appear to have been confirmed experimentally, and represents a gap in knowledge.

Serum amyloid A (SAA), like CRP, is a blood marker of systemic inflammation in humans. It is found in low-levels in humans, and has shown potential similar to CRP as a marker for cardiovascular risk. However, the availability of human assays for SAA is very limited, making the clinical potential for use of this marker poor (Pearson, 2003). In animal studies, SAA is known as the mouse-homologue for CRP. This means that it plays an equivalent physiological role to CRP in humans but in mice (Saber, A.T., 2009). SAA assays for mice are readily available, and SAA is known as a significant marker for *in vivo* studies of the cardiovascular system using mouse models.

3.3.2 Pro-thrombotic Proteins Fibrinogen and soluable P-selectin

Fibrinogen is an acute phase protein that is linked to cardiovascular risk; however, unlike CRP and SAA, fibrinogen has direct pro-thrombotic effects. Fibrinogen is a precursor, which, in the presence of thrombin, converts to fibrin in the coagulation cascade. Insoluble fibrin is central to platelet aggregation and blood clot formation (thrombosis). Another molecule involved in thrombosis is P-selectin. P-selectin is an adhesion molecule that is understood to assist in the rolling of leukocytes through the vasculature and eventually into the tissues. The adhesion properties of P-selectin act to aggregate platelets to each other, to leukocytes, and to the vascular endothelium (Ay, C., 2007). In order for platelets to become aggregated into a clot, platelet activation must occur. One method of determining platelet activation is by confirming the presence of P-selectin. P-selectin is translocated to the cell surface of activated platelets. A portion of the P-selectin is released into the blood in soluble form (sP-selectin). Activated vascular endothelial cells also translocate and release P-selectin under inflammatory conditions.

(Cascio, 2007). These prothrombotic effects can contribute to the conditions where cardiovascular morbidity and mortality may increase.

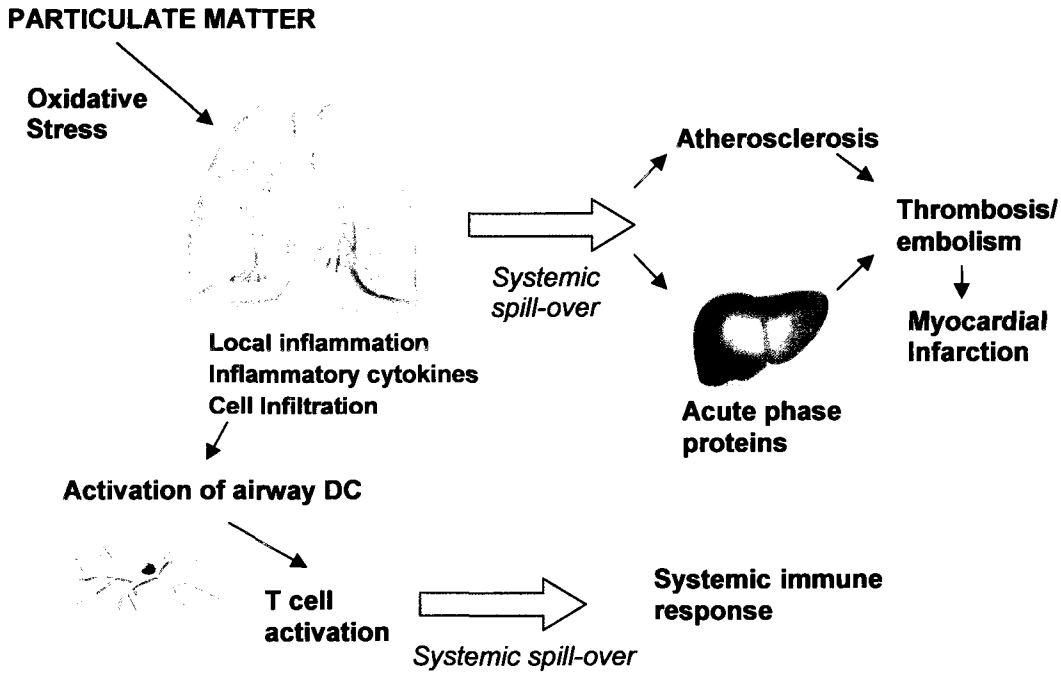

Figure 3:

Effect of particulate matter inhalation on local inflammatory and systemic inflammatory responses; relation of systemic spill-over to cardiovascular effects.

4.0 Study Model

We have used a mouse model of PM inhalation in order to investigate the acute pulmonary and systemic effects of exposure to air pollution *in vivo*. *In vivo* studies allow researchers to evaluate the effects of treatment on a complete physiological system involving different cell types and molecules of interest simultaneously, and allows for the

interpretation of system interactions. Mice are a common study animal for air pollution studies, and Balb/c mice are often used in studies of allergic disease (Ritz, S.A., 2004; Stampfli, M.R., 1998; Nilsen, A., 1997; Li, N., 2010). Diesel exhaust particles (DEP) are frequently used as a model for particulate air pollution (Diaz-Sanchez, D., 1999; Diaz-Sanchez, D., 1996; Manzo, N.D., 2009; Rudell, B., 1999; Singh, P., 2004; Salvi, S., 1999; Takizawa, H., 2004).

4.1 Diesel Exhaust Particles

For the current study, DEP generated by the operation of a forklift were obtained from the National Institute of Standards and Technology (Reference Material 2975). Diesel exhaust particles are composed of a carbon core with components such as metals and polyaromatic hydrocarbons present on the particle surface. Diesel exhaust particles are a major component of air pollution in urban, industrial, and traffic-heavy areas, accounting for up to 90% of ultrafine particulate mass in major cities of the world (Salvi, S., 1999). Until recently, diesel combustion was thought to be more environmentally friendly than gasoline because of better fuel efficiency. However, diesel emissions generate up to 100 times more particles than similar-sized gasoline engines (Salvi, S., 1999). Diesel is widely used to fuel engines of various types, including personal and commercial vehicles and small and large engines from generators to heavy industrial equipment (United States Environmental Protection Agency, 2009).

4.2 Exposure Method

The exposure method chosen for the current study is intranasal instillation. In this method, DEP in solution is applied drop-wise to an anaesthetized animal *in vivo*, and naturally inhaled (Southam, D.S., 2002). This method is known to be safe and non-invasive; it requires no surgery, and very short-term anesthetic. Compared to chamber exposures, dose of DEP is more easily controlled in this method. Intranasal instillation is a realistic exposure method because DEP are captured in the upper and lower respiratory mucosae, mimicking natural inhalation.

Objectives

In this study we investigated the effects of pulmonary and systemic inflammation *in vivo* after exposure to DEP. Pulmonary inflammation was measured by observing changes in cell infiltration, activation and cytokine secretion. Secondly, we investigated the effect of DEP on systemic inflammation. Gaps in knowledge remain around the potential for activation of dendritic cells by DEP, and the generation of acute phase proteins as an indication of a systemic response to DEP.

This study will help us understand some of the broad mechanisms linking particulate matter and the aggravation of allergic respiratory and cardiovascular conditions. With increasing urbanization, and an aging population in Canada, it is wise to understand the potential health impacts of the contaminated air we breathe.

Materials and Methods

Particles

Diesel exhaust particles (DEP) were obtained from the National Institute of Standards and Technology (Standard Reference Material 2975; Gaithersburg, MD). Particles were suspended in 40 ml of sterile phosphate buffered saline (PBS) at a concentration of 16.67 μg/ml and sonicated in a tightly capped vessel for 2 minutes. Dilutions of this stock were prepared in sterile containers under a biological safety hood in order to limit the possibility of contamination.

Animals

Female Balb/c mice were obtained from Charles River (Montreal, QC, Canada) at 6-8 weeks of age. Mice were housed in a dedicated room with a 12 h light/dark cycle and an ambient temperature of 20-21°C. Mice were housed 4 per cage in sterile cages with irradiated corncob bedding (Harlan Laboratories, Indianapolis, IN). Irradiated food (PMI Nutrition International, Brentwood, MO) and autoclaved water was available *ad libitum*. Animal experiments were approved by the Laurentian University Animal Care Committee under animal use protocol number 2006-01-03.

Intranasal Instillation of DEP

Mice were anaesthetized with isoflurane and treated by intranasal instillation of DEP suspended in sterile PBS. DEP was administered by pipette in two treatments, 15 µl each. Each 15 µl portion was ejected dropwise onto the nostril of the mouse and inhaled as the mouse breathed. After treatment, mice were allowed to recover in their home cage.

Euthanasia and Tissue Collection

At various time points after DEP treatment, mice were anaesthetized with isoflurane and killed by exsanguination. Blood (about 250-500 µl) was obtained by cardiac puncture, and stored in a Capiject tube with gel clot activator (Terumo Medical Products, Elkton, MD) on ice. In addition, about 10 µl of blood was collected in a heparin-coated capillary tube (Chase Scientific Glass Inc., Rockwood, TN) and transferred to a microcentrifuge tube. Spleens were isolated, and lungs were removed with trachea and heart attached. Lung perfusion was performed to remove blood from the lung vasculature by inserting a needle into the right ventricle of the heart, and perfusing approximately 20 ml of Hank's Balanced Salt Solution (HBSS) [Sigma-Aldrich, St. Louis, MO] through the heart to the lungs. Heart and trachea were cut away, and discarded. Lungs and spleens were stored on ice in HBSS.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed by cannulating the trachea with polyethylene tubing, securing the trachea around the tubing by tying with nylon thread, and infusing the lungs with two volumes of PBS. To infuse the lungs, a needle was inserted into the tubing and 0.25 ml of PBS was discharged from a graduated syringe. The lungs were tapped gently 60 times, and BAL fluid was withdrawn and kept on ice. This step was repeated with another 0.20 ml volume of PBS, and added to the first volume on ice. BAL viable cell counts were performed using 0.4% Trypan Blue dye (EMD Chemicals Inc., Gibbstown, NJ) and a haemocytometer. The BAL sample was spun down at 14,000 rpm for 15 seconds, and supernatant removed and stored at -20°C for future cytokine analysis by ELISA. The cell pellet was resuspended in 100 µl of PBS, and cell smears were prepared by cytocentrifugation (Shandon Inc, Pittsburgh, PA) at 300 rpm for 2 minutes. Smears were allowed to dry overnight and then stained with Diff-Quik (Thermo Scientific, Waltham, MA) according to product directions.

Differential cell counts were performed on BAL smears on at least 300 BAL cells using standard criteria to identify mononuclear cells, neutrophils, and eosinophils. Endocytosis of diesel particles was assessed by a semi-quantitative scoring regime: Mononuclear cells and neutrophils were scored as 'no diesel' if there was no evidence of endocytosis of DEP; Cells were counted as having endocytosed a significant amount of DEP if more than 25% of the area within the cell membrane appeared black.

Serum, Plasma and Peripheral Blood Smears

Blood collected in Capiject tubes was allowed to come to room temperature and then spun down at 14000 rpm for 10 minutes. The serum and plasma was collected and stored at -20°C. Blood smears were prepared from the unclotted sample, and stained as per BAL smears.

ELISAs

Granulocyte macrophage colony stimulating factor (GM-CSF) in mouse BAL fluid was measured by commercially-available ELISA (BD OptEIA Set Mouse GM-CSF, BD Biosciences, San Diego, CA.) according to product protocol. The limit of detection for the ELISA was 16.6 pg/ml. Determination of serum amyloid A (ALPCO, Salem, NH), and sP-selectin (R&D Systems, Minneapolis, MN) in mouse serum was similarly assessed by ELISA according to product protocol. Limits of detection for these assays were 31.25 ng/ml and 0.31 ng/ml, respectively. Determination of fibrinogen in mouse plasma was assessed by commercially-available ELISA (ALPCO, Salem, NH) with a detection limit of 25 ng/ml.

Lung Cell Isolation

Lungs were stored in HBSS at 4°C overnight. The next day, lungs were sliced into 4 mm sections and immersed in 10 ml of 150 U/ml collagenase (Worthington Biochemical, Lakewood, NJ) solution per set of lungs. Samples were incubated in a 37°C water-bath with shaking for 1 hour. Afterwards, collagenase solution and lungs were poured through a 40 μm cell strainer, and cells were dispersed by pressing the tissue

pieces through the strainer with the plunger from a 5 ml syringe. The collagenase and lung cells were recovered and spun down at 1,200 rpm for 10 minutes, after which cells were resuspended in 10 ml of staining buffer and spun down again at 1,200 rpm for 10 minutes, and the pellet resuspended in 0.5-1 ml of staining buffer. Lung cell counts were performed using Trypan blue dye and a haemocytometer, and adjusted to a concentration of $20x10^6$ cells/ml.

Spleen Cell Isolation

Due to the high prevalence of cell surface markers of interest, splenocytes were used as compensation controls for each fluorochrome measured by flow cytometery. One spleen was collected per experiment. The spleen was stored in HBSS at 4°C overnight. The next day, excess HBSS was poured off into a waste container and the spleen was placed in a petri dish. Fatty tissue was removed from the spleen using a scalpel, and the spleen was sliced into 4 mm sections. Fresh HBSS was added to the petri dish , and spleen sections were lightly ground between the frosted faces of two glass slides. Spleen suspension was filtered into a 50 ml tube through a 40 μm nylon cell strainer (BD Biosciences, Mississauga, ON). The filtered splenocyte suspension was centrifuged for 10 minutes at 4°C and 1,500 rpm. Supernatant was removed and the pellet mixed by gentle tapping. One ml of ACK lysis buffer (0.15M NH_4Cl, 1mM $NaHCO_3$, 0.01mM EDTA) was added to the tube and mixed by pipette for 1 minute; 9 ml of HBSS was added and mixed gently to end the lysis. The tube was centrifuged again for 10 minutes at 4°C and 1,500 rpm. The cell pellets were resuspended in staining buffer. Viable cells

were counted using Trypan Blue and a haemocytometer, and the concentration of cells was adjusted to $20x10^6$ cells/ml.

Lung and Spleen Cell Staining for Flow Cytometry

Fc Block (BD Pharmingen, San Jose, CA) was added to each tube of lung and spleen cells at a concentration of $1\mu g/10^6$ cells, and incubated on ice for 20 minutes. After incubation, 50 µl of the cell suspension ($1x10^6$ cells) was distributed to individual 5 ml polystyrene tubes.

Antibody concentrations for staining were determined by titration using splenocytes to maximize signal and limit background staining with the isotype control (data not shown). Anti-mouse antibodies utilized were obtained from BD Pharmingen (San Jose, CA) and included: Biotin-conjugated rat-anti-mouse anti-CD40 (3/23); APC-conjugated rat-anti-mouse anti-CD25 (3C7); FITC-conjugated rat-anti-mouse anti-CD3 (17A2); PE-conjugated hamster-anti-mouse anti-CD69 (H1.2F3); Biotin-conjugated rat-anti-mouse anti-B7-2 (GL1); PE-conjugated hamster-anti-mouse anti-CD54 (3E2); APC-conjugated hamster-anti-mouse anti-CD11c (HL3); FITC-conjugated rat-anti-mouse anti-MHCII (2G9); PE-conjugated hamster-anti-mouse anti-B7-1(16-10A1); PerCP Cy5.5-conjugated rat-anti-mouse anti-CD4 (RM4-5); PE Cy7-conjugated rat-anti-mouse anti-CD8 (53-6.7). In addition, appropriate isotype controls were selected for each of the antibodies, also obtained from BD Pharmingen.

Antibodies were added to tubes and allowed to incubate on ice for 20 minutes, protected from light. After incubation, 2 ml of staining buffer (1% BSA in PBS) was added to each tube and mixed by pipette. Tubes were centrifuged for 5 minutes at 1,300

rpm and 4°C. Supernatant was removed, and cells were washed two more times for a total of three washes. After last wash, secondary staining (PE-Cy7 conjugated streptavadin) was applied where appropriate (BD Pharmingen, San Jose, CA). Stained cells were resuspended in 1 ml of staining buffer. Tubes were kept on ice, protected from light, and analyzed immediately on BD Facs Canto II flow cytometer.

Statistical Analysis

Sigma Stat version 2.03 software was used for statistical analysis. Differences were considered statistically significant when P□0.05 by ANOVA. Where normality failed, Kruskal-Wallis Analysis of Variance on Ranks was performed.

Results

Internalization of DEP by MNC and Neutrophils

The effects of intranasal instillation of DEP in mice was observed in mouse lungs after harvest. Deposition and accumulation of DEP in the lungs was macroscopically evident, increasing in intensity with dose (Figure 4, A-D). Gross examination of the lungs suggests that although there are areas where the particles are more heavily concentrated than others (likely at major branching points of the respiratory tree), DEP are dispersed widely through the lungs after intranasal instillation. Observation of cells isolated from BAL fluid showed internalization of DEP by mononuclear cells at doses of 10 μg, 50 μg or 250 μg of DEP (E-H). The internalization of DEP by cells on BAL slides was semi-qualitatively quantified by counting the number of MNC and neutrophils that internalized treatment particles. The % MNC with internalized DEP increased significantly for all times points in groups given 50 μg and 250 μg of DEP vs. vehicle alone ($p < 0.05$). In groups given 10 μg of DEP, there was no difference from control (I-L). The % neutrophils with internalized DEP trended similarly. At all time points, there was significantly more neutrophils with internalized DEP in groups given 250 μg vs. vehicle. Groups given 50 μg DEP had significantly more neutrophils with internalized

DEP at 12 h and 24 h, but no difference from control at 6 h. In groups given 10 μg of DEP the internalization of particles was not significantly different from control (M-N).

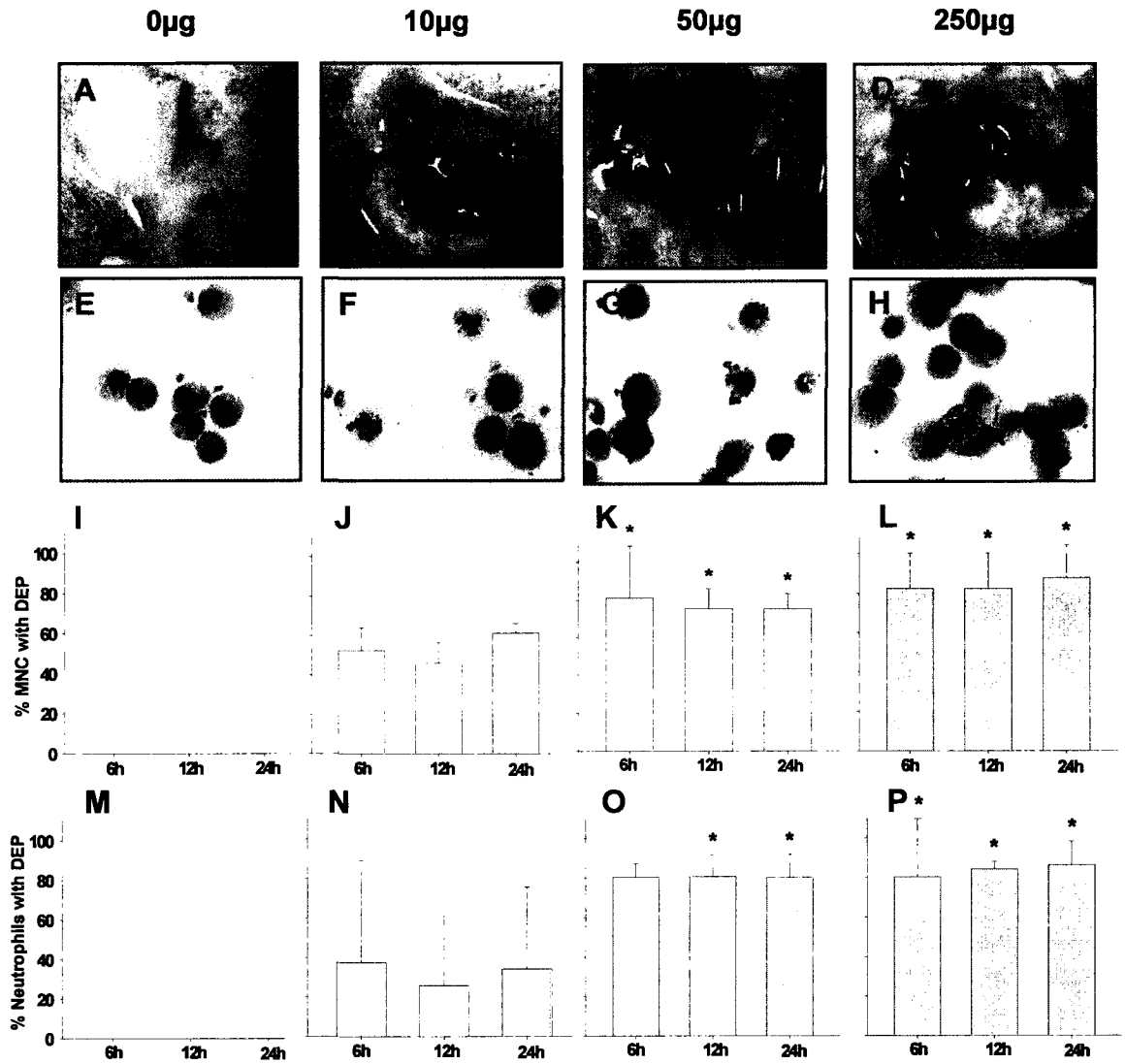

Figure 4:

Internalization of DEP in mouse lung. Dose response and time course of treatment with DEP (10, 50, 250 µg) or control (0 µg). Panels A-D show representative photos of harvested lungs. Panels E-H show representative photos of BAL slides. Panels I-L show % mononuclear cells on BAL slides with internalized DEP at 6, 12 and 24 h after treatment. Panels M-P show % neutrophils on BAL slides with internalized DEP at 6, 12 and 24 h. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in % of cells ($p < 0.05$) compared to control at the same time point.

Cell infiltration into the lungs after DEP treatment: TCN, MNC, neutrophils

As a measure of airway inflammation, changes in leukocyte infiltration were assessed in response to treatment with DEP. There was a significant increase in TCN at 6 h and 24 h in BAL obtained from mice given 250 μg DEP vs. vehicle alone ($p < 0.05$) (Figure 5). At 12 h, there was no difference between the treatment groups. Proportions of MNC and neutrophils in a population of cells were compared on BAL slides, and it was noted that, with DEP treatment, as the proportion of MNC decreased, neutrophils increased (Figures 6 and 7). Specifically, the % MNC significantly decreased with 250 μg DEP treatment vs. vehicle alone ($p < 0.05$) at every time point. Similarly, the number of MNC per ml of BAL fluid significantly decreased at 12 h with 250 μg DEP treatment vs. vehicle ($p < 0.05$), while no differences were recorded at 6 h and 24 h (Figure 6). In the opposite trend, the % neutrophils significantly increased at doses of 50 μg and 250 μg of DEP compared to vehicle ($p < 0.05$) at all time points. Similarly, the number of neutrophils per ml of BAL fluid significantly increased at doses of 50 μg and 250 μg of DEP compared to vehicle ($p < 0.05$) at all time points (Figure 7).

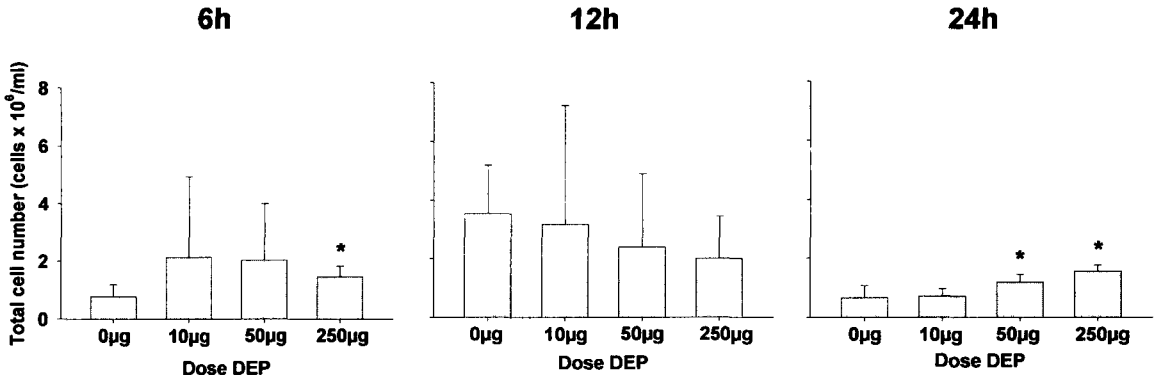

Figure 5:

Total inflammation in the lung 6, 12, and 24 h after DEP treatment (10 µg, 50 µg, 250 µg) or control (0 µg). Data are shown as means +/- standard deviation (n=10-12). * indicates a significant difference in total cell number ($p < 0.05$) compared to control at same time point.

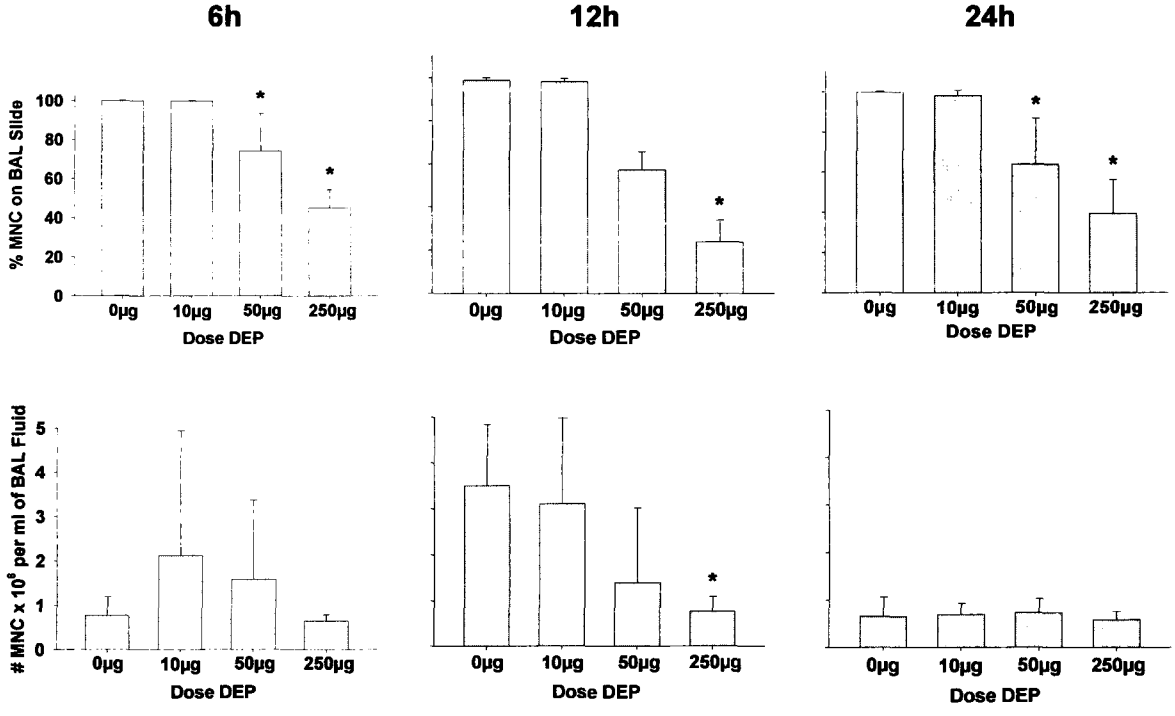

Figure 6:

Mononuclear cells in the lungs after treatment with DEP (10 µg, 50 µg, 250 µg) or control (0 µg). Data are shown as % of cells counted, and number of cells per ml of BAL fluid. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control at same time point.

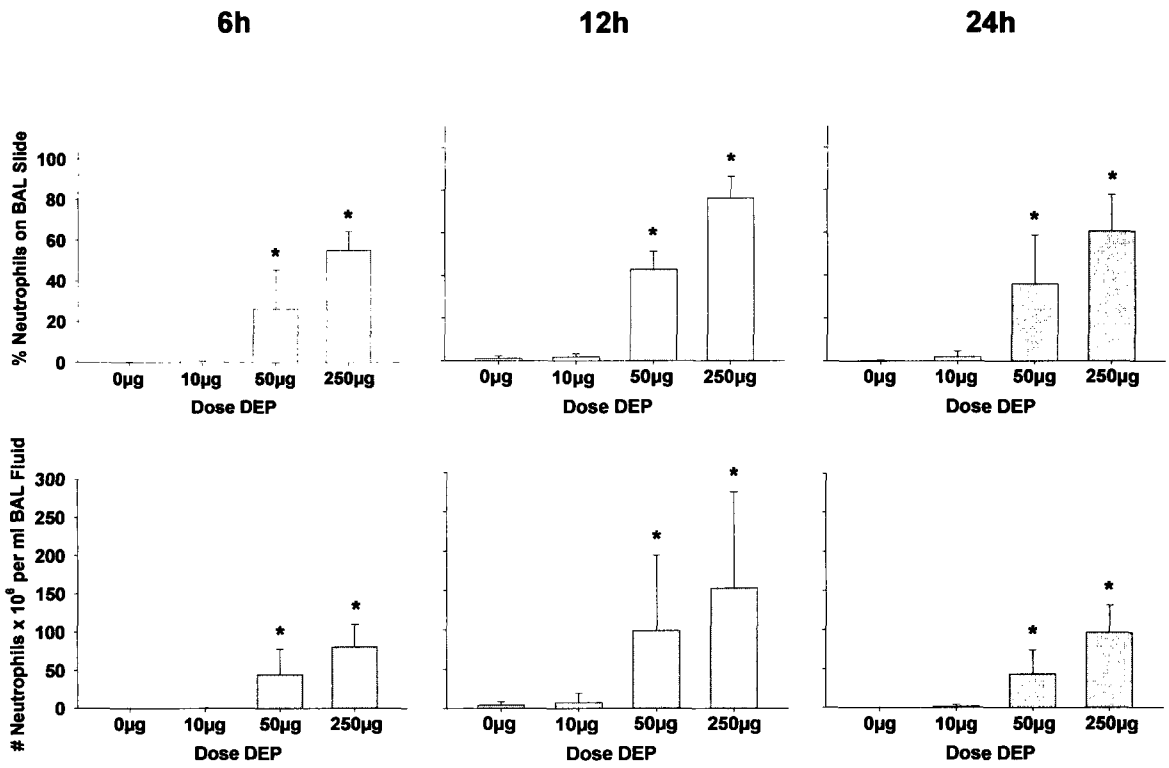

Figure 7:

Infiltration of neutrophils into lungs after treatment with DEP (10 µg, 50 µg, 250 µg) or control (0 µg). Data are shown as percent of cells counted, and number of cells per ml of BAL fluid. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control at same time point.

Cell infiltration into the lungs after DEP treatment: APCs

Changes in antigen presenting cell infiltration were assessed in response to treatment with DEP by flow cytometry. At 6 h, there was a significant increase in the number of macrophages in the lungs of mice treated with 250 μg DEP vs. vehicle alone ($p < 0.05$), while no changes were seen at 12 h and 24 h ($p=0.034$) (Figure 8). Similarly, at 6 h there was a significant increase in the number of dendritic cells in the lungs of mice treated with 250 μg DEP vs. vehicle alone ($p < 0.05$), while no changes were seen at 12 h and 24 h (Figure 9).

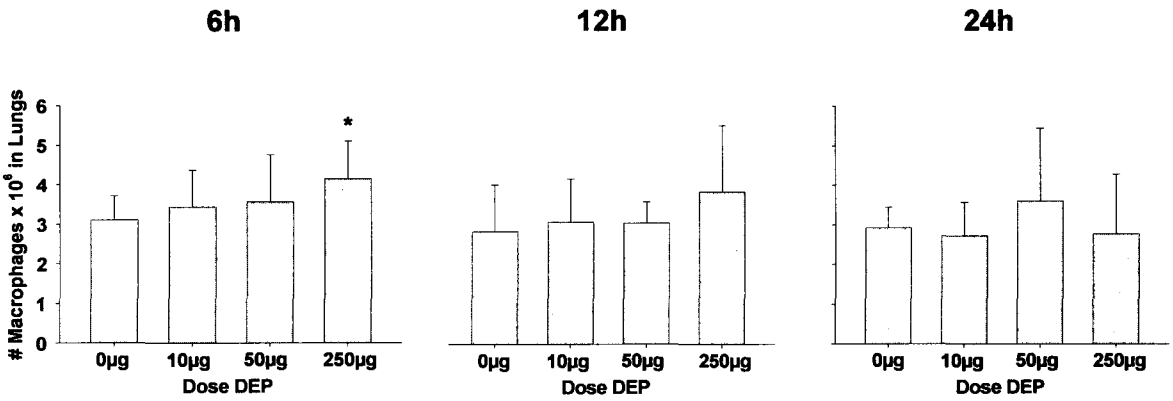

Figure 8:

Number of macrophages (MHCII+/CD11c-) in lung tissue after treatment with DEP (10 µg, 50 µg, 250 µg) or vehicle (0 µg) as determined by flow cytometric analysis. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control at same time point.

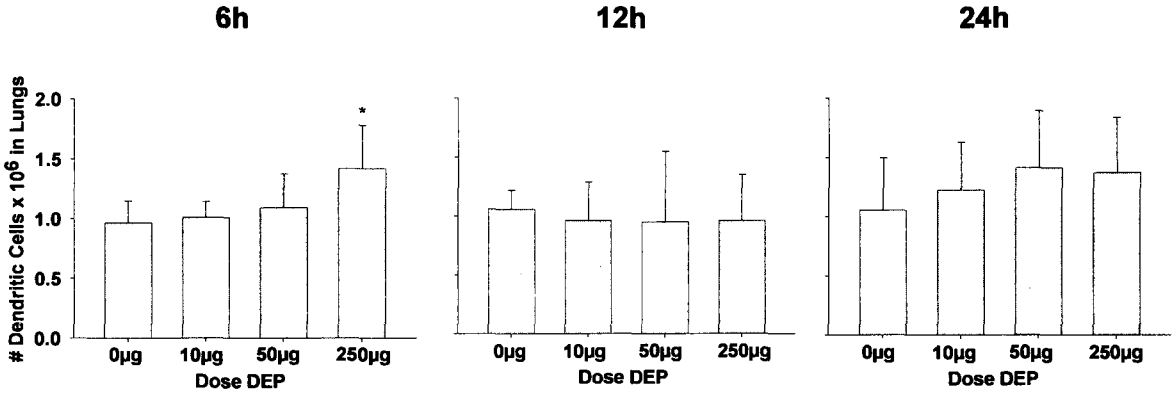

Figure 9:

Number of dendritic cells (CD11c+/MHCII+) in lung tissue after treatment with DEP (10 µg, 50 µg, 250 µg) or vehicle (0 µg) as determined by flow cytometric analysis. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control at same time point.

Expression of CD54 by APC

Expression of activation molecule CD54 by antigen presenting cells was assessed in response to treatment with DEP. No significant changes in CD54 expression were noted on macrophages in response to treatment at any time point ($p=0.073$) (Figure 10). However, changes in CD54 expression were noted on dendritic cells. At 6 h, there was a significant increase in the expression of CD54 by dendritic cells in mice treated with 250 μg DEP vs. vehicle alone ($p < 0.05$). At 12 h, no changes were observed between treatment groups. At 24 h, a significant increase in expression of CD54 by dendritic cells was observed at doses of 50 μg and 250 μg of DEP compared to vehicle ($p < 0.05$) (Figure 11).

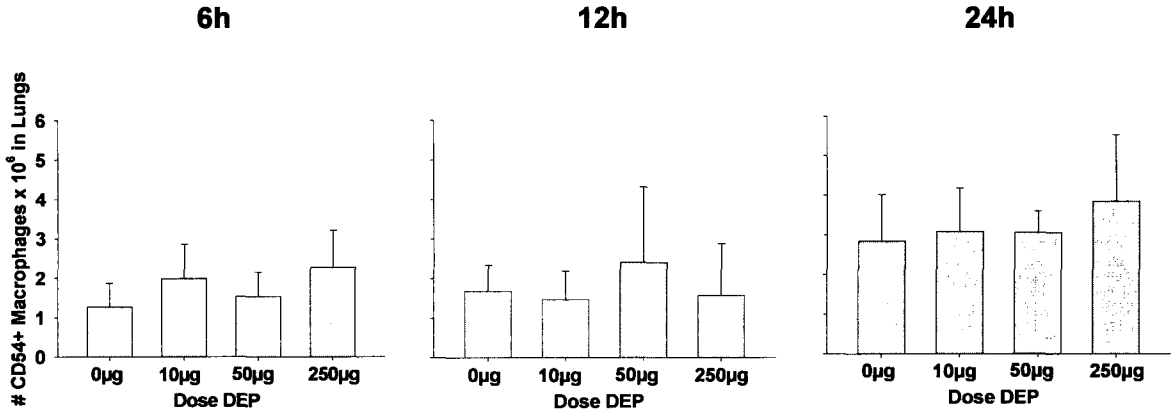

Figure 10:

Number of CD54+ macrophages (CD11c-/MHCII+) in lung tissue after treatment with DEP (10 µg, 50 µg, 250 µg) or control (0 µg) as determined by flow cytometric analysis. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control.

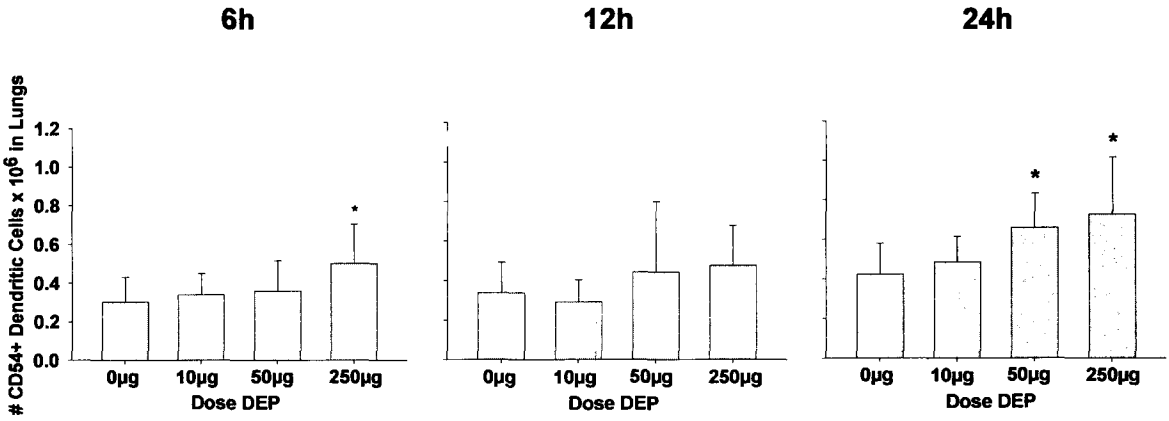

Figure 11:

Number of CD54+ dendritic cells (CD11c+/MHCII+) in mouse lung tissue after treatment with DEP (10 µg, 50 µg, 250 µg) or vehicle (0 µg) as determined by flow cytometric analysis. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference in number of cells ($p < 0.05$) compared to control at same time point.

Presence of GM-CSF in BAL fluid

Presence of GM-CSF was assessed in mouse BAL fluid after treatment with DEP. At 6 h and 12 h, significant increases in GM-CSF were observed in groups treated with 50 μg and 250 μg of DEP compared to vehicle ($p < 0.05$). No changes were observed between groups 24 h after treatment ($p=0.866$) (Figure 12).

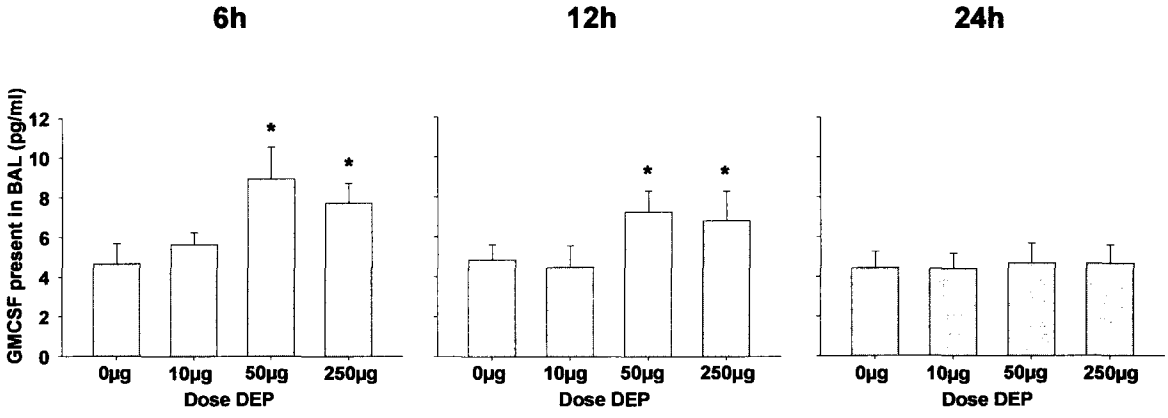

Figure 12:

Concentration of granulocyte-macrophage colony stimulating factor (GM-CSF) in mouse BAL fluid collected after treatment with DEP (10 μg, 50 μg, 250 μg) or control (0 μg) as determined by ELISA. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference ($p < 0.05$) compared to control at same time point.

Acute phase response to DEP treatment

Acute phase proteins were measured in mouse serum or plasma after treatment with DEP. At 6, 12, and 24 h after treatment, significant increases in SAA in mouse serum were observed in groups treated with 250 μg of DEP compared to vehicle ($p < 0.001$) (Figure 13). sP-selectin levels were similarly assessed in serum. At 6 h, there were significant decreases in sP-selectin levels in groups treated with 50 μg and 250 μg of DEP compared to vehicle ($p < 0.019$). No changes were observed between DEP-treated groups and vehicle at 12 h. 24 h after treatment, there was a significant decrease in sP-selectin levels in serum in the group treated with 10 μg of DEP compared to vehicle ($p < 0.001$) (Figure 14). Plasma fibrinogen levels at were assessed 12 h after treatment with vehicle or 250 μg of DEP and no significant changes were observed ($p=0.327$) (Figure 15).

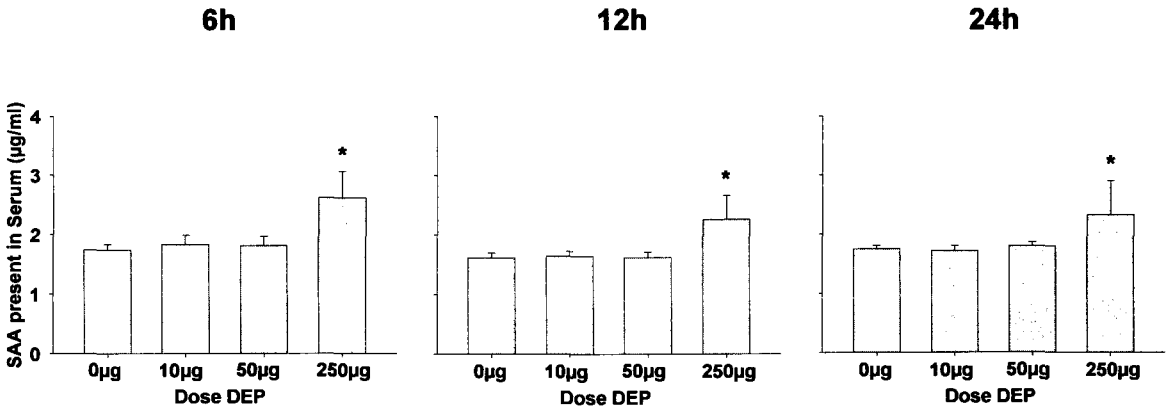

Figure 13:

Concentration of serum amyloid A (SAA) in mouse serum after treatment with DEP (10 µg, 50 µg, 250 µg) or control (0 µg) as determined by ELISA. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference ($p < 0.05$) compared to control at same time point.

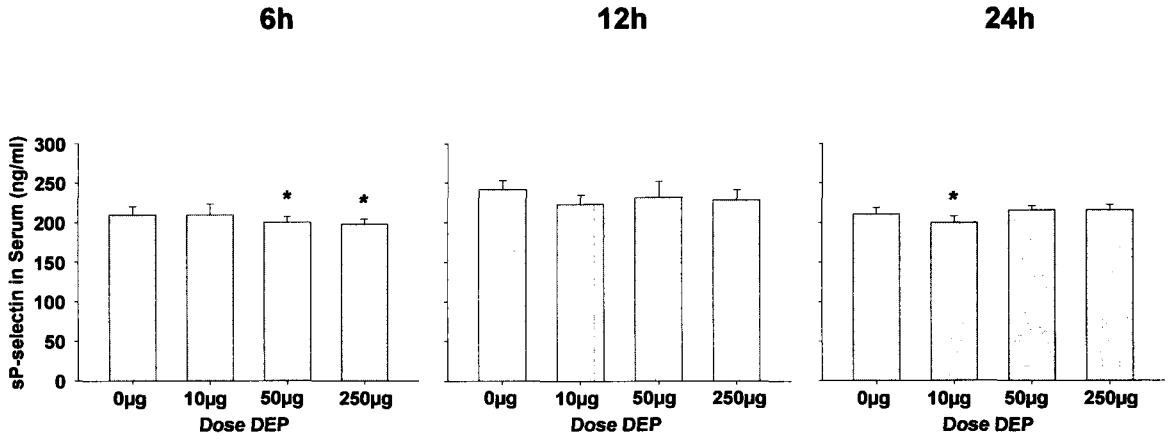

Figure 14:

Concentration of sP-selectin in mouse serum after treatment with DEP (10 µg, 50 µg, 250 µg) or control (0 µg) as determined by ELISA. Data are shown as means +/- standard deviation, n=10-12. * indicates a significant difference ($p < 0.05$) compared to control at same time point.

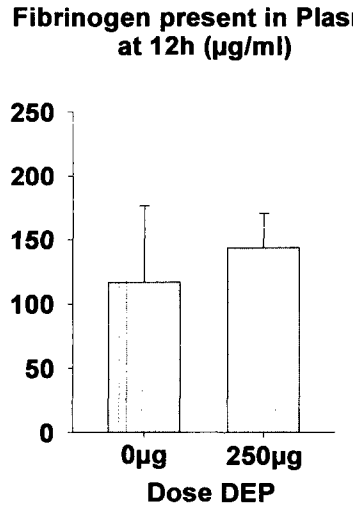

Figure 15:

Concentration of fibrinogen in mouse plasma 12h after treatment with DEP (250 µg) or control (0 µg) as determined by ELISA. Data are shown as means +/- standard deviation, n=6. * indicates a significant difference ($p < 0.05$) compared to control at same time point.

Discussion

Delineating the molecular and cellular mechanisms linking exposure to air pollution with adverse health outcomes will be crucial to the development of strategies to mitigate these effects. In this study, we have investigated the events occurring in the airways and systemically after respiratory exposure to DEP; notably, we have demonstrated an increase in APC infiltration into the lungs after exposure, and documented evidence of the induction of an acute phase response. These findings have significance for our understanding of how exactly inhalation of PM causes respiratory and cardiovascular symptoms.

Relevance of Exposure Method: Intranasal Instillation of Particles as a Model for Inhalation Exposure

Epidemiological studies have long shown a link between air pollution and detrimental effects to health. Experimental *in vitro* and *in vivo* studies have been designed to investigate epidemiological trends and further explore the mechanisms behind them. Exposure regimes have been established that utilize different models for inhalation exposure. While particles, doses and exposure methods vary among studies, some commonalities have been identified. Particles commonly used in studies include

diesel exhaust particles and ambient particles from various sources (Cascio, W., 2007; Cozzi, E., 2007; Diaz-Sanchez, D., 1999; Inoue, K., 2008; Mamessier, E., 2006; Manzo, N.D., 2009; Mutlu, G., 2007; Nilsen, A., 1997; Rudell, B., 1999). Diesel exhaust particles are readily available and standardized, and represent a significant portion of particulate matter pollution in urban areas (Salvi, S., 1999). For this reason, diesel exhaust particles are commonly used in research, including the current study. A documented method of DEP exposure in *in vivo* studies is intranasal instillation. This route is non-invasive and is shown to be effective in mice. In this method, a volume of a solution of liquid plus treatment particles is applied to the nares of a mouse, and naturally inhaled. Suspended particles reach the lungs and make contact with lung epithelia, mimicking natural inhalation. Analysis shows that approximately 20% of treatment administered will reach the lungs when a delivery volume of 10ul is administered (Southam, D.S., 2002). Consistent with this observation, gross examination of lungs from mice treated with DEP by intranasal instillation in our study showed that DEP reached the lungs.

While intranasal instillation is not as similar to natural inhalation as exposure to particles in a chamber suspended in air, it allows more control over the dose of particles administered. This control allows researchers to consider if the dose is a relevant comparison to epidemiological findings the study may intend to explore. Doses of DEP used in experimental pollution studies often vary from 10 μg to 400 μg (Southam, D.S., 2002) Commonly, and consistent with our work, doses of 50 μg and higher are associated with inflammatory effects in mice (Singh, P., 2004; Southam, D.S., 2002; Stoeger, T., 2006; Inoue, K., 2008; Ichinose, T., 2004). Doses administered in the current

study were based on pollution studies found in literature and estimations of particulate matter exposure relevant to human health. Calculations using worldwide peak PM concentrations and comparisons of minute-ventilation values for mice and humans suggest that the 50 μg dose in our study is comparable to a day spent in a large city in the USA, such as Los Angeles, CA, and the 250 μg dose is comparable to a day spent in one of the world's 'megacities', such as Mexico City, Mexico (Mutlu, G., 2007).

Effect of PM on Inflammation and Allergy

Allergic respiratory diseases, such as asthma and allergic rhinitis are characterized by hyper-responsiveness to stimuli, IgE-secreting B-cells, mast cells, histamine release and mucus secretion. Mediators of the allergic inflammatory process include inflammatory cytokines, chemokines, and cells of the immune system such as APC and T cells. The effect of reactive oxygen species on the allergic response is being explored and gaining recognition. This recognition is in part due to studies indicating that particulate matter, which is shown to be an effective inducer of oxidative stress, can impact inflammatory allergic responses.

When particulate matter is inhaled, it reacts with molecules on cells of the airway epithelia, producing reactive oxygen species. The generation of oxidative stress impacts redox sensitive transcription factors that mediate inflammation. The result is increases in inflammatory cytokines which are implicated in inflammatory immune responses.

The impact that PM is shown to have on allergic responses is an adjuvant effect. Studies show that in experimental models of allergy, increased neutrophil and eosinophil infiltration, and IgE and cytokine secretion are associated with particulate matter

treatment (Granum, B., 2001; Diaz-Sanchez, D., 1996; Miyabara, Y., 1998). One of the proposed explanations for these findings is the pro-inflammatory effect of PM. Another, which the current study investigates, is the effect of PM on dendritic cells.

Dendritic cells are important mediators in the development of allergy. Located in the airway epithelia, they survey the airway for inhaled antigens and signs of threat. Encountered antigens are internalized, processed and peptides are presented on the cell surface. At this point, the dendritic cell migrates to the lymph node to present the antigen to T cells (Banchereau, J., 2000). The current study found that in mice treated with DEP there was a significant increase in the number of DC in the lungs. This indicates that DC infiltrated from nearby tissues in response to stimuli generated by DEP treatment. To our knowledge, we are the first group to quantify a significant increase in dendritic cells in the lungs after treatment with particulate matter. Other groups have demonstrated dendritic cell recruitment into the airways associated with bacterial peptides and inflammatory cytokines (McWilliams, A., 1996; Stumbles, P.A., 2001). Increased numbers of dendritic cells would be expected to increase the efficiency of antigen capture in the airway. More antigens could potentially be encountered and subsequently presented to T cells. The obvious result here would be a greater antigen load being delivered to T cells, with the potential for greater numbers and potency of immune responses against the antigens. If the antigens are allergens, this would mean increased possibility of allergic responses.

Of course, in order for a DC to activate a T cell against a presented antigen, it must express the required co-stimulatory molecules on its surface. The expression of co-stimulatory molecules on DC is mediated by recognition of pathogen associated

molecular patterns, and signals from inflammatory cytokines (Sabatte, J., 2007). An immune response will not be generated in absence of these molecules, specifically B7.1 and B7.2, characteristic of a mature DC. If particulate matter inhalation can induce secretion of inflammatory cytokines in the airway, for instance by epithelial cells (Singh, P., 2004; Miyabara, Y., 1998; Stoeger, T., 2006; Takizawa, H., 2004), one would expect that PM might induce maturation in DC, particularly if cytokines known to stimulate DC maturation (such as GM-CSF) are produced. One could reasonably expect that this would have an impact on the development of allergic sensitization.

In vitro studies appear to show that dendritic cells can be stimulated to mature by particulate matter, particularly when cultured with bronchoepithelial cells (Porter, M., 2007; Bleck, B., 2006). However, GM-CSF is used in the cultivation of DC *in vitro*, and, GM-CSF is known to induce maturation in DC. Therefore, it is unclear if GM-CSF is stimulating maturation, even in apparent 'no treatment' groups, and particulate matter is acting as an adjuvant to the process. In addition, while these studies measure accessory maturation molecules, such as CD40, they do not measure the key T cell co-stimulatory molecules B7.1 or B7.2.

Comparable *in vivo* studies of dendritic cell maturation and particulate matter are rare. Inoue et al (2008) measured B7.1 and B7.2 expression *in vivo* on DC after treatment with vehicle, OVA, 100 μg DEP, or DEP in combination with OVA. Results showed that there was no difference in B7 expression on DC treated with DEP or vehicle; significant increases in B7's were identified in mice treated DEP and OVA together, and OVA alone (Inoue, K., 2008). No other dendritic cell maturation markers were

investigated in this study. In absence of antigen, dendritic cell maturation was not confirmed.

We attempted to measure the expression of co-stimulatory molecules B7.1 and B7.2 on dendritic cells but were unable to due to technical problems. Interestingly, we are the first group to show a significant increase in the maturation marker CD54 on dendritic cells in mice treated with DEP. The expression of CD54 enhances the adhesion between the dendritic cell and the T cell, providing increased opportunity for antigen detection and T cell stimulation. This novel finding suggests that in absence of any particular antigen treatment, dendritic cells in mice are functionally activated via expression of CD54 after exposure to diesel exhaust particles. Further studies are required to replicate the current findings and further explore the effect of PM on DC *in vivo*.

In our context, the significance of expression of maturation markers by dendritic cells in mice exposed to particulate matter lies in fleshing out the mechanistic links between particulate matter and allergy and asthma. The consequence of activation, and potentially, full maturation of dendritic cells in concert with particulate matter is the enhancement of the adaptive immune response. *In vitro* and *in vivo* experiments demonstrate that particulate matter acts as an adjuvant to allergen sensitization and allergic response (Takizawa, H., 2004; Diaz-Sanchez, D., 1999; Granum, B., 2001; Ichinose, T., 2004; Miyabara, Y., 1998). Studies report that production of IgE and cytokines are enhanced when allergens are encountered in association with PM vs. allergen alone (Diaz-Sanchez, D., 1997; Anonymous, 1999). The enhanced function of DC in association with PM is potentially one of the key mechanisms to explain these

findings. In addition, the enhanced ability of dendritic cells to stimulate T-cells is a potential mechanism for the finding that particulate matter *induces* sensitization to new antigens in atopic individuals. (Diaz-Sanchez, D., 1999). The enhancement of antigen presentation by dendritic cells in association with DEP, in concert with increased cytokine secretion, would function to facilitate sensitization to antigens present in the airways. This means that atopic individuals exposed to PM will be more likely than those exposed to clean air to develop new allergies to new antigens that have not bothered them previously.

PM, the Acute Phase Response and Associations with Cardiovascular Risk

While there is a logical link between the danger of inhalation of particulate matter and respiratory health, the link between air pollution and cardiovascular health is not as immediately obvious. However, as epidemiological and experimental data unravels the broad physiological effects of inflammation, the link is becoming clearer.

As discussed, exposure to particulate matter causes an inflammatory response in the lungs. This local inflammation has the capacity to become systemic. The production of cytokines by local cells spills over into circulation, where effects can be seen in the bone marrow and liver (Peters, A., 2001; Suwa, T., 2002; Van Eeden, S.F., 2002; Wilson, D.W., 2010). This effect is important in the recruitment of cells to sites of infection, but, when there is no infectious agent present, the intense inflammation can be detrimental.

The acute phase response occurs when the liver responds to stimuli by upregulating the production of a variety of proteins that support host defense; the presence of these proteins is a marker of systemic inflammation. CRP and fibrinogen are

acute phase proteins secreted by the liver during the acute phase response. CRP is thought to have a role in the destabilization of atherosclerotic plaques, and fibrinogen is a prothrombotic protein that increases the potential for blood clots to form (Burke, A.P., 2002; Cao, Jie J., 2003; Levenson, J., 1995). Both of these proteins are well established markers of cardiovascular risk and have been shown to be elevated in epidemiological studies investigating particulate matter and cardiovascular health (Brook, R.D., 2004; Donaldson, K., 2001; Pope, C. A. III, 2004; Peters, A., 2001).

In mice, the homologue for C reactive protein is serum amyloid A (SAA) (Saber, A.T., 2009). Our study showed a significant increase in SAA at all time points in mice treated with 250 μg of DEP vs. control. As far as we can tell, increased SAA (or CRP in humans) in response to particulate matter has never before been demonstrated experimentally (although increases in CRP have been associated with exposure to PM in epidemiological studies) (Pope, C. A. III, 2004; Peters, A., 2001). Our result showing significant increases in SAA in mice in response to DEP is a strong experimental link between cardiovascular risk, systemic inflammation and particulate matter.

Although not replicated in the current study, experimental studies have uncovered significant increases in the acute phase marker fibrinogen, and the pro-inflammatory molecule sP-selectin in mice exposed to particulate matter (Mutlu, G., 2007; Cozzi, E., 2007; Medeiros, N., Jr., 2003; Cascio, W., 2007). Our results showed either no change, or a decrease in these markers. Comparable mouse studies used similar time points (24 h), particle preparation (PM suspended in PBS or saline), and doses of particulate matter (100 μg). The major difference noted was the type of particle. Our study utilized DEP, while comparable studies used ambient particles as treatment; thus we speculate that the

difference in chemical composition may potentially account for the disparity in these findings.

Ambient particles are heterogeneous, unlike DEP, and have the potential for greater toxicity. Particle size, composition, surface area and reactivity are important in their potential toxicity. In particular, fine particulates featuring adsorbed metals and polyaromatic hydrocarbons have well established toxicity (Duffin, R., 2002; Prieditis, H., 2002; Tran, C., 2000). McCartney et al (2009) compared the carbon, polyaromatic hydrocarbon, and metal composition of ambient particles and DEP. Results showed that DEP had significantly higher carbon content (including polyaromatic hydrocarbons), while the ambient samples had higher metal content (McCartney, K., 2009). It is possible that the particles used in our study exerted different toxic effects than ambient particles at the same dose. In higher doses, it is possible that DEP would exert similar toxic effects to ambient particulate matter; this theory is backed up by a study that found significant increases in plasma fibrinogen 24 h after exposure to 400 μg of DEP (Inoue, K., 2005), which is almost twice the dose of DEP that we used.

Another possibility is that fibrinogen and sP-selectin proteins in the blood in our study may have degraded before analysis by ELISA. sP-selectin in plasma is understood to be unstable, sometimes degrading within hours after collection (Lafrenie, R., personal communication). Similarly, plasma fibrinogen is known to be unstable at room temperature, making it a more difficult marker to accurately measure in studies of cardiovascular disease (Pearson, T.A., 2003). While blood samples collected from study mice were kept on ice where possible, processing times for blood and tissue harvest were lengthy, leaving room for the possibility of protein degradation.

In order to resolve these issues, further study would be required, with different doses of DEP in comparison with ambient particulates. However, given that SAA was induced by DEP exposure, our findings suggest at least a partial induction of the acute phase response, indicative of systemic inflammation, was observed in mice treated with DEP.

Atherosclerosis and Acute Myocardial Infarction

Inflammation and the induction of the systemic response are implicated in cardiovascular effects such as atherosclerosis and heart attack. While precise pathways have not been established, it is theorized the presence of pro-inflammatory cytokines, and pro-thrombotic proteins contribute to biochemical conditions that increase cardiovascular risk (Peters, A., 2001; Cozzi, E., 2007) at least in part by promoting atherosclerosis, and in the destabilization of existing atherosclerotic plaques (Cao, Jie J., 2003; Suwa, T., 2002).

The initial formation of atherosclerotic plaques occurs in the vascular intima. Plaques are predominantly formed by collections of inflammatory leukocytes, especially macrophages, and the uptake of cholesterol lipoproteins. Assisting in this process are inflammatory cytokines which expand white blood cell and macrophage populations. The inflammatory response also enhances the movement of cells from the tissue into the blood by endothelial cell activation and increased vascular permeability (58 Donaldson, K., 2001). Particularly in high risk groups, these inflammatory processes have the potential to accelerate the development of atherosclerotic plaques. Our findings affirm that exposure to PM can cause the release of soluble factors, such as GM-CSF and SAA,

that can promote these processes. Indeed, Suwa et al (2002) completed an *in vivo* study investigating the links between particulate matter and atherosclerosis. This study found that the progression of atherosclerotic plaques was increased in mice treated with particulate matter vs. control. In addition, cellular infiltration and the induction of the systemic response were significantly increased in animals exposed to particles (Suwa, T., 2002).

The existence of plaques and thickening of blood vessel walls increases the risk of acute cardiovascular events. Myocardial infarctions are often preceded by the rupture of an atherosclerotic plaque. An existing plaque, specifically the cap of the plaque, may be induced to rupture through the release of enzymes by activated neutrophils and macrophages. These enzymes, commonly released during inflammatory responses, have the capacity to break down the fibrous structure of the plaque (Pearson, T.A., 2003). The release of the atherosclerotic plaque cap opens up the core of the plaque to the blood. At this point the core is exposed to platelets, fibrin, and red blood cells, making the conditions ripe for the formation of a blood clot. From here, two avenues are possible; the first, and arguably the more desirable avenue, is the possibility that the blood clot dissolves the plaque, and the plaque and clot are safely cleared. The other possibility is that the blood clot will grow, cause vascular blockage, and subsequent heart attack. This catastrophic pathway is more likely to result if biochemical influences such as those factors investigated in the current study (CRP/SAA, fibrinogen, sP-selectin), are present. (Toole, J.F., 1988) Our study confirms experimentally that inhalation of PM can lead to the upregulation of factors, such as SAA, that promote this kind of process.

Future Directions

Studies investigating the effect of particulate matter on local and systemic inflammation continue to be relevant. Of particular interest to our group is the activation and potential full maturation of dendritic cells after PM exposure. As discussed, the effect of DEP on expression of B7 molecules by DC was not successfully incorporated into the current study. *In vivo* explorations of this area remain under-investigated in literature. The role of DC in the mediation and development of allergy, especially in the context of PM exposure, remains to be fully elucidated. An interesting avenue to pursue in this respect would be a comparison of DEP and ambient particle exposure on DC maturation status *in vivo*, given the disparities in their chemical composition. This experiment would reveal if there are differing effects of ambient particle vs. DEP exposure on DC. The current study demonstrated that in the absence of antigen, CD54 expression was significantly increased in mice exposed to DEP. If CD54 expression is increased, it is possible that B7 molecules could be as well. The implications of full maturation of DC by PM exposure in a natural setting could be potently enhanced allergic sensitization to any number of antigens encountered in ambient air. Further investigations will flesh out these mechanisms and the possible implications on allergic respiratory health.

The effect of PM exposure on systemic inflammation remains a theory to support observations of acute exacerbations of cardiovascular disease. The current study showed significant increases in SAA in mice exposed to DEP. However, we were not able to replicate increases in fibrinogen and sP-selectin that other groups have generated. As discussed, it is possible that the choice of study particle or dose may have played a role in our results. Particles sourced from the Paris subway system, or from a nickel mining operation, or from the operation of a diesel-fueled forklift, have varied compositions and toxicities, and may generate similarly variable inflammatory effects. Differences between particles and potential cardiovascular effects remain to be elucidated. This represents a potential source of contention with current studies in this field.

Conclusions

Our results have shown significant biological effects associated with respiratory and cardiovascular diseases in mice treated with DEP. We have shown significant increases in the inflammatory cytokine GM-CSF, expanding populations of white blood cells such as neutrophils and macrophages, significant increases in dendritic cells in the lungs, the induction of the maturation marker CD54, and significant increases in the acute phase protein serum amyloid A. These results help explain increased symptoms of allergic airway diseases and incidence of cardiovascular events on days of poor air quality. A better understanding of the impact of contaminated air will inform public health and environmental strategies, and improve the health of Canadians for generations to come.

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